Delayed treatment with sildenafil enhances neurogenesis and improves functional recovery in aged rats after stroke

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Abstract

Increasing age decreases the number of new neurons in the dentate gyrus and the subventricular zone (SVZ). Sildenafil, a phosphodiesterase type 5 (PDE5) inhibitor, enhances neurogenesis in young rats. The present study tested the hypothesis that sildenafil augments neurogenesis in aged rats after stroke. Non-stroke aged (18 month, n=6) Wistar rats exhibited a significant reduction of actively proliferating and relatively quiescent cells in the SVZ measured by the number of minichromosome maintenance protein-2 positive (MCM-2$^+$) cells, a marker of the proliferating cells, compared with non-stroke young (3-4 month, n=8) rats. Occlusion of the middle cerebral artery (MCA) did not increase the number of MCM-2$^+$ cells in the SVZ of aged rats at 3 months after stroke. However, treatment with sildenafil at a dose of 3 mg/kg (n=8) daily for 7 consecutive days starting 7 days after stroke significantly increased the number of MCM-2$^+$ cells in the SVZ of aged rats compared with aged rats treated with saline (n=8). Many of the proliferating cells were doublecortin positive, a marker of migrating neurons. In addition, treatment with sildenafil significantly improved functional recovery compared with saline treated rats. These data suggest that inhibition of PDE5 activity by sildenafil evokes neurogenesis in the SVZ of aged stroke rats, although these rats have reduced in numbers of neural progenitor and stem cells in the SVZ.

Introduction

The subventricular zone (SVZ) of the adult rodent contains actively proliferating progenitor cells and relatively quiescent stem cells, which generate neurons and glia throughout adulthood (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Morshead et al., 1998). Stroke increases neurogenesis in the SVZ of young adult rats and newly generated neurons migrate towards ischemic boundary regions (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003a; Zhang et al., 2003; Zhang et al., 2004b; Zhang et al., 2004a). However, the majority of new neurons do not survive in the ischemic boundary over the time (Arvidsson et al., 2002; Zhang et al., 2004b). Phosphodiesterase type 5 (PDE 5) enzyme is highly specific for hydrolysis of cGMP (Corbin and Francis, 1999). Administration of sildenafil, a specific inhibitor of PDE5, substantially increases cGMP levels in the brain and enhances neurogenesis in the SVZ of young rats after stroke (Zhang et al., 2002b). Aged rats exhibit a decrease in the basal levels of cGMP as a consequence of an increase in phosphodiesterase activity (Chalimoniuk and Strosznajder, 1998) and neurogenesis in the dentate gyrus and SVZ declines with increasing age (Seki and Arai, 1995; Tropepe et al., 1997; Maslov et al., 2004). However, it is not known whether cGMP levels in aged animals have neurogenic effects as in the young animals. Studies on aged animals could have important clinical implications for stroke treatment since stroke is a major cause of death and disability in the elderly (Paolucci et al., 2003). Accordingly, we investigated the effects of sildenafil on neurogenesis in aged rats after stroke.

Materials and Methods

All experimental procedures have been approved by Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal model of stroke: Male Wistar rats at ages of 3-4 and 18 months were classified as

young adult and aged, respectively. The right middle cerebral artery (MCA) was occluded by placement of a 24 h old fibrin-rich clot at the origin of the MCA (Zhang et al., 1997). Briefly, a modified PE-50 catheter with a 0.3mm outer diameter filled with a single clot was gently advanced from the right external carotid artery (ECA) into the lumen of the internal carotid artery (ICA) nearby the origin of the MCA. The clot was then injected along with 2-3 μl of 0.9% saline.

Experimental Protocol: 1) Actively and slowly proliferating cells in the SVZ express MCM-2 in mice (Maslov et al., 2004). To examine if MCM-2 expression can be detected in cells that proliferate slowly within the SVZ of rats, cytosine-β-D-arabinofuranoside (Ara-C), an anti-mitotic drug that kills the majority of proliferating progenitor cells, (2%, Sigma-Aldrich, St. Louis, MS) in vehicle (0.9%, saline) was continuously infused onto the surface of the right hemisphere of the young rats (n=6) for 7 days starting on the day of MCA occlusion with a mini-osmotic pump (Alzet, Palo Alto, CA model 2001; at rate of 1.0 μl/hr, 7 D) (Doetsch et al., 1999; Zhang et al., 2004b). Cannulas were implanted on the brain surface at anterior / posterior, 0, 1, and 1.1 mm relative to the bregma (Zhang et al., 2004b). Young stroke rats (n=6) without infused with Ara-C were as the control group. Bromodeoxyuridine (BrdU, 100 mg/kg), which is incorporated into the DNA of dividing cells during S-phase, was injected (I.P.) every 2 hr for a total of 6 hr on the day prior to sacrifice. Rats were killed 7 days after MCAo and immunostaining with antibodies against MCM-2 and BrdU was performed. 2) To examine the effect of age on proliferating cells in the SVZ, aged (n=6) and young (n=8) non-stroke rats were sacrificed and the number of MCM-2$^+$ in the SVZ was measured. 3) To examine the effect of delayed treatment with sildenafil on stroke, sildenafil, which has a half-life of 0.4h in rats (Walker et al., 1999), was administered (IP) at a dose of 3 mg/kg (n=10) to aged rats daily for 7

consecutive days starting 7 days after MCA occlusion. Aged stroke rats (n=10) were treated with the same volume of saline as the control group. These rats were sacrificed 12 weeks after stroke.

Functional Outcome: An array of behavioral tests were performed on the aged rats at pre-stroke, 4 and 7 days after stroke and then weekly for 11 consecutive weeks after stroke.

Neurological severity scores (NSS): NSS is a composite of motor, sensory, reflex, and balance tests (Chen et al., 2001). Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).

Adhesive removal test: An adhesive removal test was employed to measure somatosensory deficits (Schallert and Whishaw, 1984). The mean time required to remove both stimuli from limbs was recorded.

Foot-Fault test: Rats were tested for forelimb placement dysfunction with the foot-fault test (Zhang et al., 2002a). Data are presented as the numbers of left forelimb foot-faults divided by the total numbers of steps (movement of each forelimb), expressed as a percentage.

Histology and Immunohistochemistry: At the end of the experiment, animals were transcardially perfused with heparinized saline followed by 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde. Infarct volume was measured on 7 hematoxylin and eosin (H&E) stained coronal sections using a Global Lab Image analysis program (Data Translation, Marlboro, MA)(Zhang et al., 1997).

Immunohistochemistry was performed, as previously described (Zhang et al., 2001). The following antibodies were used in the present study: mouse anti-BrdU antibody (1:1000, Boehringer Mannheim, Indianapolis, IN), goat anti-MCM-2) antibody (1:300, Santa Cruz Biotechnology Inc. Santa Cruz, CA), goat anti-doublecortin (DCX) antibody (1:200, Santa Cruz

Biotechnology), rabbit anti-phospho-Histone H3 (HH3) antibody (1:1000, Upstate Biotech, Lake Placid, NY), and rabbit anti-Ki67 antibody (1:300, Abcam, Cambridge, MA).

Using the Dako EnVision Doublestain System (Dako), double immunostaining for BrdU and MCM-2 was performed on paraffin embedded coronal sections according to the manufacture's instructions. Adjacent sections were double stained with antibodies against Ki67 and HH3 or Ki67 and DCX.

For measurement of MCM-2$^+$ cells, every 60th MCM-2 immunostained coronal section at the level of AP + 10.6 mm and AP + 9.2 mm was digitized under a 60x objective (Olympus BX40) via the MCID computer imaging analysis system (Imaging Research, St. Catharines, Canada). Labeled cells along the SVZ of the lateral ventricular wall were counted on a computer monitor to improve visualization and in one focal plane to avoid over-sampling. MCM-2$^+$ cells in the SVZ were presented as the number of the cells/section. The density of MCM-2$^+$ cells in the 4 sections per rat was averaged to obtain a mean density value for each brain according to published methods (Kuhn et al., 1996; Zhang et al., 2001).

Statistical analysis One way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was used. The data are presented as means±SE. A value of $p<0.05$ was taken as significant.

Results

Two aged rats in the saline treated group died at 5 and 8 days after stroke, while two aged rats in the sildenafil group died at 5 and 6 days after stroke prior to the treatment. These rats were excluded from further evaluation.

MCM-2 expression in the SVZ

MCM-2 is required for all dividing cells and is expressed in actively proliferating and relatively

quiescent SVZ cells of adult mouse brain (Maiorano et al., 1996; Maslov et al., 2004). To examine the distribution of MCM-2$^+$ cells in the SVZ of rat, double immunostaining for BrdU and MCM-2 was performed in young stroke rats. Many SVZ cells were both BrdU$^+$ and MCM-2$^+$, while some SVZ cells were either BrdU$^+$ only or MCM-2$^+$ only (Fig. 1A). To verify that MCM-2$^+$ is expressed in the relatively quiescent SVZ cells, Arc-C was infused to young stroke rats for 7 days, which kills the majority of actively proliferating cells (Doetsch et al., 1999; Zhang et al., 2004b). Immediately after termination of Ara-C infusion, BrdU$^+$ cells disappeared, while MCM-2$^+$ cells were still detected in the SVZ (Fig. 1B), suggesting that MCM-2$^+$ identifies relatively quiescent SVZ cells. Thus, MCM-2 is expressed in actively proliferating and relatively quiescent SVZ cells in rats. Accordingly, immunostaining of MCM-2 was used for examining the SVZ cell population in the aged rats.

Sildenafil enhances neurogenesis in the aged rats

Analysis of MCM-2$^+$ in the SVZ revealed that non-stroke aged rats had a significant reduction in the number of MCM-2$^+$ cells (Fig. 2B and Table 1) compared with the number in young adult rats (Fig. 2A and Table 1). Consistent with reduction in MCM-2$^+$, aged rats also exhibited a significant decrease of the number of Ki67$^+$ and HH3$^+$ cells (Table 1), a marker of G_2/M phases of diving cells (Hendzel et al., 1997). Three months after stroke, the number of MCM-2$^+$ cells in the SVZ of aged rats did not increase significantly (19.48 ± 0.36) compared with non-stroke aged rats (19.02 ± 0.29). However, treatment with sildenafil significantly increased the number of MCM-2$^+$, Ki67$^+$ and HH3$^+$ cells (Fig. 3B and Table 1) compared with the number in the saline treated group 3 months after stroke (Fig. 3A and Table 1). Double immunostaining revealed that more Ki67$^+$ cells were DCX$^+$ (Fig. 3D) in sildenafil treated than in saline treated aged animals (Fig. 3C) indicating that treatment with sildenafil increases neurogenesis in aged rats.

Delayed administration of sildenafil improves neurological function in aged rats

Our previous study demonstrated that administration of sildenafil 24h after stroke enhances functional recovery in aged rats (Zhang et al., 2005). To examine the effects of delayed administration of sildenafil on functional recovery on aged rats, we administered sildenafil 7 days after stroke and measured functional recovery over 3 months. An array of behavioral tests including NSS (Fig. 4A), foot-fault (Fig.4B), and adhesive removable test (Fig. 4C) revealed that treatment with sildenafil significantly improved neurological function starting 30 days after stroke which persisted for 3 months. Treatment with sildenafil 7 days after stroke did not significantly change infarct volume compared with saline treated animals (Fig. 4D).

Discussion

The present study demonstrates that aged rats exhibit a significant reduction of the number of MCM-2$^+$ and Ki67$^+$ cells in the SVZ compared with young rats, yet treatment with sildenafil 7 days after stroke significantly increased the number of MCM-2$^+$ and Ki67$^+$cells in the SVZ in aged rats. Many of Ki67$^+$ cell were DCX$^+$, indicating increases of neurogenesis. In addition, treatment of aged rats with sildenafil 7 days after stroke substantially enhanced functional recovery. These data suggest that aging decreases neurogenesis in the SVZ, while sildenafil enhances neurogenesis in aged rats and improves neurological outcome after stroke.

Aging decreases neurogenesis in the dentate gyrus and the SVZ in the rodent (Seki and Arai, 1995; Kuhn et al., 1996; Jin et al., 2003b; Maslov et al., 2004). In the present study, we found substantial reduction of neurogenesis in the SVZ of aged (18 moth-old) rats compared with young adult rats. However, Kuhn et al did not detect a reduction of the number of BrdU cells in the SVZ of the aged rat (21 month-old), although they found a significant decrease of neurogenesis in the dentate gyrus (Kuhn et al., 1996). The use of different markers to identify

proliferating cells in the SVZ could offer one plausible explanation for the discrepancy between the present result and the finding of Kuhn. The SVZ contains actively proliferating progenitor cells and relatively quiescent stem cells (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Morshead et al., 1998). The dose of BrdU, paradigms of BrdU injection and times of sacrificing animals after BrdU injection affect the labeling index of SVZ cells (Nowakowski et al., 1989; Takahashi et al., 1993; Dolbeare, 1996). The present study used markers of MCM-2, Ki67, and HH3 to measure proliferating cells in the SVZ of the aged rat. MCM-2 is required for all dividing cells and is expressed in progenitor and stem cells in the SVZ of the adult mouse (Maslov et al., 2004). Consistent with findings in the mouse, we found that MCM-2$^+$ cells were present in the SVZ of the young adult rat after depletion of actively proliferating progenitor cells by the Ara-C. Aged rats exhibited significant reductions not only in the number of cells reactive for both MCM-2$^+$ and Ki67$^+$ but also in the number of cells reactive for only MCM-2$^+$ in the SVZ, suggesting that the present observation of reduction in neurogenesis in the aged rat could be attributed to decreases in the number of progenitor and stem cells, which is consistent with findings in the aged mouse (Maslov et al., 2004).

Stroke induces neurogenesis in young adult and aged rodents (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003a; Jin et al., 2004; Zhang et al., 2003; Zhang et al., 2004a; Zhang et al., 2004b). The current study did not find that stroke increased neurogenesis in the SVZ of the aged rat when neurogenesis was measured 3 months after stroke. Stroke-induced neurogenesis peaks 7 days after stroke and decreases thereafter (Arvidsson et al., 2002; Zhang et al., 2004a; Zhang et al., 2004b). Thus, it is possible that the stroke-induced neurogenesis in the SVZ subsides in the aged rat 3 months after stroke. By contrast, treatment with sildenafil significantly increased neurogenesis in the aged rat, indicating that the aged brain has the capacity to generate new

neurons, which is consistent with previous findings (Jin et al., 2004). Sildenafil is a specific inhibitor of PDE5 which degrades cGMP (Corbin et al., 1999). The endogenous basal level of cGMP mediates neurogenesis in the neural progenitor cells derived from the SVZ of young adult rat (Wang et al., 2005). Sildenafil increases cGMP levels in the neural progenitor cells and enhances neurogenesis in young adult rats (Zhang et al., 2002b; Wang et al., 2005). The cellular signals which reduce neural progenitor and stem cells in the aged brain are largely unknown. In the aged brain, the basal level of cGMP is substantially reduced by increased PDE activity (Chalimoniuk and Strosznajder, 1998). These data, together with the present findings, suggest that decreased cGMP levels could contribute to age-related decrements in neurogenesis.

Aged rats exhibit a greater impairment of neurological recovery compared with young rats after stroke (Borlongan et al., 1995; Badan et al., 2003; Lindner et al., 2003). Decreased brain plasticity including synaptogenesis, angiogenesis and neurogenesis could contribute the impairment of functional recovery (Badan et al., 2004; Zhang et al., 2005). Treatment of stroke with sildenafil 24 h after stroke enhances angiogenesis, synaptogenesis, and neurogenesis, which is coincident with substantial improvement of functional recovery in young and aged rats (Zhang et al., 2002b; Zhang et al., 2005). The present study demonstrates that delayed (7 days after stroke) administration of sildenafil improved functional recovery in aged rats and significant recovery was not observed until 30 days after stroke compared with saline treated rats. Therefore, age-related functional impairment after stroke could be reversed through enhancement of brain plasticity.

In summary, the present study demonstrates that neurogenesis in the SVZ of aged rats decreases compared to young rats and sildenafil augments neurogenesis in stroke brain and improves functional recovery in aged rats.

Figure Legends:

Fig. 1 Infusion of Ara-C eliminates actively proliferating cells in young rats. Panels A and B are images of double immunostaining of BrdU (red) and MCM-2 (brown) in the SVZ from a representative rat 7 days after stroke (A) or a rat treated with Ara-C 7days after stroke (B). Black arrows indicate both BrdU and MCM-2 positive cells, whereas black arrowheads represent mitotic cells with BrdU and MCM-2 immunoreactivity. White arrows and white arrowheads indicate $BrdU^+$ only and $MCM\text{-}2^+$ only, respectively. LV = lateral ventricle and bar = 10 μm.

Fig. 2 Aged rats exhibit reduction of proliferating cells in the SVZ. Panels A and B show $MCM\text{-}2^+$ cells in the SVZ from non-stroke representative young (A) and aged (B) rats. Decreases in the number $MCM\text{-}2^+$ cells were noted in the SVZ of the aged rat (B) compared with the number in the young rat (A). LV = lateral ventricle and bar = 10 μm.

Fig. 3 Treatment of stroke with sildenafil increases neurogenesis in the SVZ of aged rats. Panels A and B show $MCM\text{-}2^+$ cells in the SVZ of representative aged rats treated with saline (A) and sildenafil (B). Panel C and D show double immunostaining for $Ki67^+$ (red) and DCX^+ (green) cells in the SVZ of the representative aged rats treated with saline (C) and sildenafil (D) after stroke, respectively. LV = lateral ventricle and Str = striatum. Bar = 10 μm.

Fig. 4 The effects of sidenafil on functional recovery on aged rats. Treatment of stroke with sildenafil improves neurological function measured by NSS (A), foot-fault test (B), adhesive removal (C). Panel D shows infarct volume measured 3 months after stroke. * and # $P<0.05$ and $P<0.01$, respectively, vs the saline group.

Table 1. The percentage of MCM-2$^+$ cells in the SVZ and the percentage of Ki67$^+$ and HH3$^+$ cell within MCM-2$^+$ cell population in young and aged rats.

Groups	MCM-2 (%)	Ki67 (%)	HH3 (%)
Young, non-stroke	31.85 ± 0.43*	22.15 ± 0.34*	0.58 ± 0.03*
Aged, non-stroke	19.02 ± 0.29	13.0 ± 0.15	0.23 ± 0.03
Aged, stroke (saline)	19.48 ± 0.36	13.22± 0.19	0.24 ± 0.02
Aged, stroke (sildenafil)	22.26 ± 0.18 #	14.85 ± 0.27 #	0.32 ± 0.02 #

For stroke rats, measurements were performed 3 months after stroke. Data are mean ± SE. * and # $P<0.05$. * vs Aged, Non-stroke group; # vs saline treated group.

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Dr. Jean de Vellis,

Enclosed, please find our manuscript entitled, "Delayed treatment with sildenafil enhances neurogenesis and improves functional recovery in aged rats after stroke"

We would appreciate your consideration for publication as an article in the Journal of Neuroscience Research.

Thank you, and I look forward to hearing from you.

Sincerely,

Michael Chopp, PhD
Professor and Vice Chairman
Department of Neurology

Thank you for your consideration.

Sincerely,

Michael Chopp, PhD
Professor and Vice Chairman
Department of Neurology

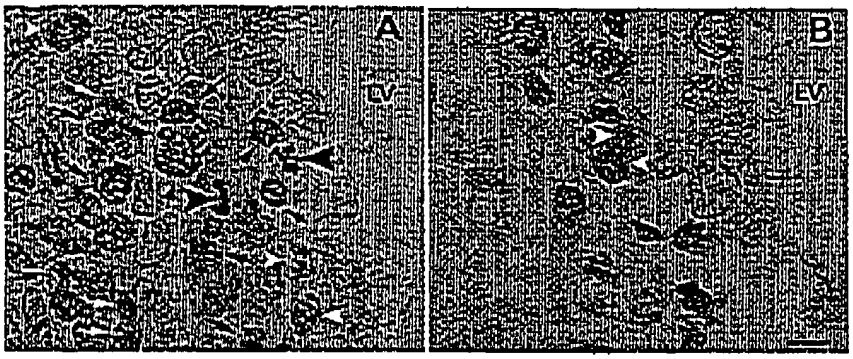

Fig. 1

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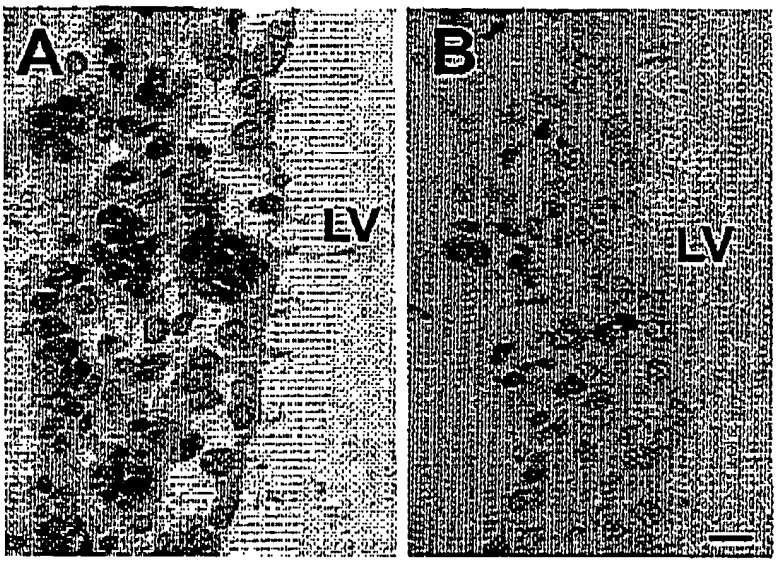

Fig. 2

127x114mm (300 x 300 DPI)

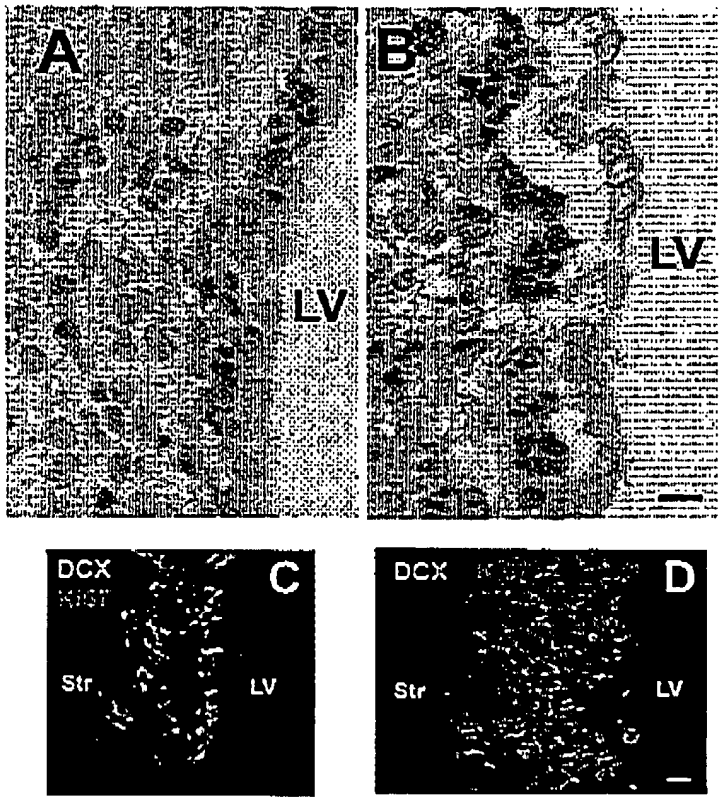

Fig. 3

127x145mm (300 x 300 DPI)

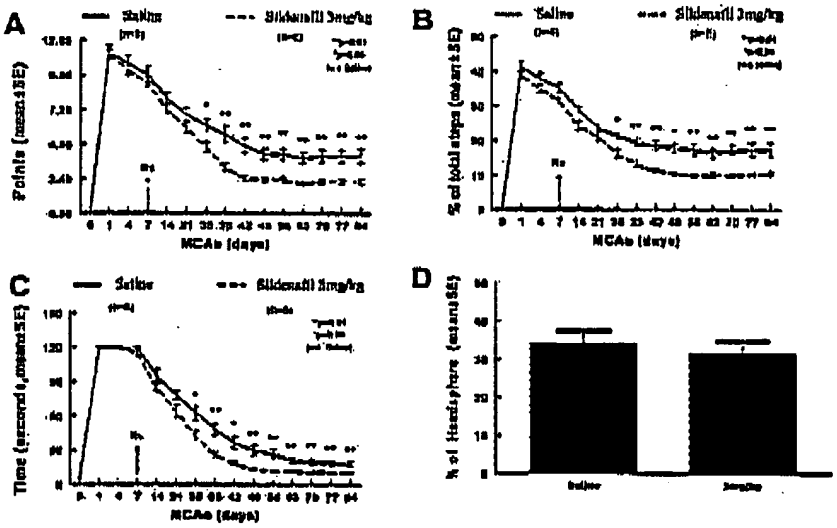

Fig. 4

203x149mm (300 x 300 DPI)